(54) Title: PREPARATION OF LARGE LIPOSOMES BY INFUSION INTO PEG

Liposome Size vs. PEG Concentration

DPPC:CHOL:DMPG 44.1:50.0:5.9
DPPC:CHOL 50.0:50.0
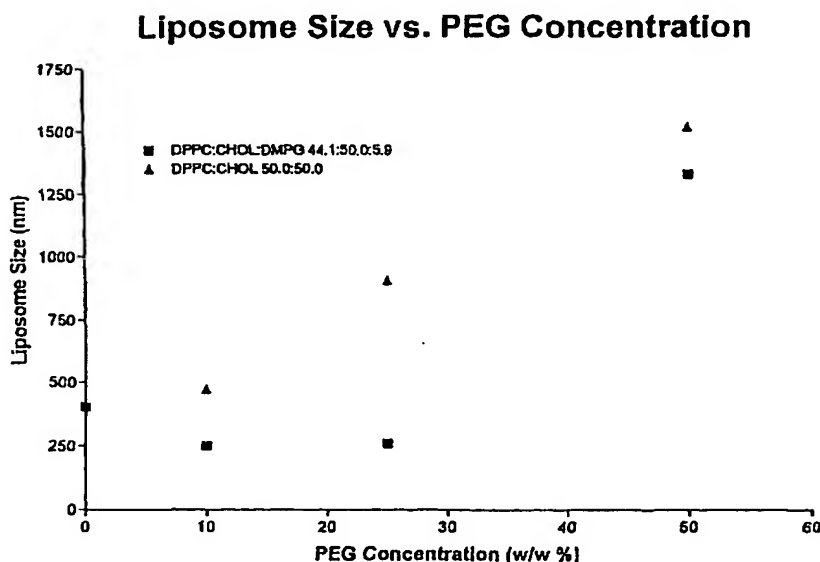
(57) Abstract: This invention provides a method of preparing large liposomes with high entrapment capacity for pharmaceutical substances. The method generally entails infusing a lipid/alcohol solution directly into an aqueous polymer solution. The resulting liposome, which may be incorporated with a pharmaceutical substance, typically consists of multilamellar vesicles that have an average size of about 1 to 8 microns. The ratio of lipid to polymer, average molecular weight of the polymer, polymer concentration, ionic strength, and temperature of incubation are variables disclosed herein to control liposome size and incorporation of components and pharmaceutical substances.

WO 02/43699 A2

WO 02/43699 A2

5

PREPARATION OF LARGE LIPOSOMES BY INFUSION INTO PEG

SUMMARY OF THE INVENTION

10   This invention provides a method of preparing large liposomes with high entrapment capacity for pharmaceutical substances.

BACKGROUND OF THE INVENTION

15   Liposomes are artificial vesicles that are completely closed lipid bilayer membranes containing an entrapped aqueous volume. Liposomes can differ in size. They can also differ in terms of lipid composition and structural organization corresponding to unilamellar (possessing a single membrane bilayer) or multilamellar (onion-like structure characterized by multiple membrane layers, each separated from the next by an aqueous layer).

20

          Liposomes have been widely used as vehicles for drug delivery. Types of liposomes used in drug delivery include small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), and multilamellar vesicles (MLVs). One of the most important characteristics that governs their use is vesicle size, which affects the rate of clearance from blood and organ

25   distribution. Liposomes between 25 nm and 10 microns have been formulated by a number of techniques to entrap pharmaceuticals.

          Based on experimental comparisons between large and small liposomes, large liposomes are cleared from the blood more rapidly than small liposomes (Poste et al., 1984;

30   Allen et al., 1983; Hunt et al., 1979). Rapid clearance is an important feature that will alter the biodistribution of liposomal entrapped drugs. Comparative studies showing greater accumulation of large liposomes have been observed in the lung (Hunt et al., 1979; Abra et al., 1984; Jackson, 1980; Fidler et al., 1980), the liver (Harashima et al., 1992; Jackson, 1980;

Allen et al., 1983), and spleen (Moghimi et al., 1991; Jackson, 1980). Large liposomes have been shown to favor uptake by Kupffer cells while small liposomes have a greater affinity to parenchyma cells (Rahman et al., 1982). Small liposomes were also shown to be capable of crossing barriers such as the sinusoidal endothelium (Poste et al., 1984). Thus, depending on

5   the target site and the pharmacokinetics of the entrapped drug, large or small liposomes may have distinct advantages.

Limitations in the current methods of making large liposomes, however, complicate their manufacture. Specifically, organic solvents (e.g., chloroform) that are commonly used

10   to ensure uniform mixing of lipid components as well as lipophilic pharmaceuticals, are highly toxic. Moreover, removal of organic solvent from the pharmaceutical preparations is difficult and tedious. Scale-up production represents a further problem for the preparation of large liposomes. In addition, commonly used methods, such as ethanol infusion, are inappropriate since the resulting product consists primarily of vesicles in the submicron range

15   (Batzri and Korn, 1973).

To overcome the above-mentioned obstacles, there is an ongoing need to prepare liposomes that are free of highly toxic organic solvents and uniformly sized in the micron range, using methods that are amenable for scaled-up production.

20

SUMMARY OF THE INVENTION

One object of the invention is to provide a method of preparing large liposomes that

25   does not require the use of highly toxic organic solvents, is cost-efficient, and is fully compatible with large-scale manufacturing. It is also an object of the invention to provide a method of preparing uniformly large liposomes, in the micron size range, that exhibit a high entrapment of both lipophilic and water-soluble pharmaceuticals. Another object of the invention is to provide a method that can be applied to the manufacture of liposomal cancer

30   vaccines suitable for biomedical applications.

In accomplishing these and other objects, there has been provided in accordance with one aspect of present invention, a method of preparing large liposomes comprising: (1) infusing a first solution, comprising at least one lipid component in a water-miscible alcohol or an alcohol/water mixture, directly into an aqueous solution of a water-soluble

5   polymer, thereby forming a liposome suspension; (2) removing the alcohol and polymer, and (3) recovering the resultant liposomes.

In a preferred embodiment, the lipid component comprises a mixture of at least one phospholipid and a sterol such as cholesterol.

10

In another embodiment, a method of preparing large liposomes further comprises adding a pharmaceutical substance to either the first solution comprising a mixture of at least one phospholipid and sterol or to the aqueous solution comprising a water-soluble polymer. The pharmaceutical substance may include a protein, a peptide, or a biological.

15

In a preferred embodiment, a cancer vaccine is provided which comprises a liposome prepared by the method of the present invention, wherein the liposome comprises a tumor antigen. In another preferred embodiment, a liposome is provided, which comprises at least one phospholipid, cholesterol, Lipid A, and a tumor antigen.

20

In yet another preferred embodiment, an infectious disease agent vaccine is provided which comprises a liposome prepared by the method of the present invention, wherein the liposome comprises an infectious disease agent antigen. Such infectious disease agent antigens can be, for example, bacteria, viruses, parasites, or a mixture thereof.

25

Both the foregoing general description and the following detailed description are exemplary and explanatory and are intended to provide further explanation of the invention as claimed. Other objects, advantages, and novel features will be readily apparent to those skilled in the art from the following detailed description of the invention.

30

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Requirements of varying PEG 6,000 concentrations by different lipid mixtures for the generation of large liposomes.

5   FIG. 2. Effect of infusion temperature on mean liposome size using 50% PEG 6,000.

FIG. 3. Effect of infusion temperature on mean liposome size using 50% PEG 20,000.

10   FIG. 4. Effect of ionic strength of the PEG solution on the final liposome size and its dependence on lipid composition.

FIG. 5. Dependence of volume and ionic strength of the PEG solution on liposome size.

15

FIG. 6. Variable cholesterol content can differentially affect the effects of PEG and saline infusions on liposome size.

FIG. 7A. Proliferation of lymph node T cells after vaccination with BLP-25

20   formulations.

FIG. 7B. Interferon-γ production by T cells from mice injected with BLP-25 formulations.

25   **DETAILED DESCRIPTION OF THE INVENTION**

The present invention relates to a novel method for preparing large liposomes with high entrapment of a pharmaceutical by infusion of a lipid-containing alcohol solution directly into an aqueous polymeric solution, such as polyethylene glycol (PEG) solution.

30

A prior art method demonstrated that small unilamellar vesicles (SUVs) will fuse to large multilamellar vesicles (MLVs) in the presence of 25-50% PEG, MW 6,000 (Hui et al., 1981; Boni et al., 1981; Boni et al., 1984). The resulting large liposomes, typically in the

micron range, were multilayered and exhibited a high entrapment of both lipophilic and water-soluble pharmaceuticals. Unlike the prior art method, the present invention circumvents the step of preparing SUVs by directly infusing a lipid/alcohol solution into an aqueous polymer solution. Furthermore, PEG, in particular, is a nontoxic water-soluble

5   polymer, available at low cost, and is FDA-approved for internal consumption (Harris, 1997; Working et al., 1997).

In one aspect of the invention, a first solution comprising a lipid component and a water-miscible alcohol or an alcohol/water mixture is provided. The lipid/alcohol solution

10   may be an emulsion, however the lipid component usually comprises a mixture of at least one phospholipid and a sterol. Preferred phospholipids are capable of forming liposomes and include phospholipids such as phosphatidylcholine (PC; lecithin). Suitable phospholipids also include phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylserine (PS). Other suitable phospholipids further include

15   dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylglycerol (DPPG), distearoylphosphatidylglycerol (DSPG), dimyristoylphosphatidylglycerol (DMPG), dipalmitoylphosphatidic acid (DPPA); dimyristoylphosphatidic acid (DMPA), distearoylphosphatidic acid (DSPA), dipalmitoylphosphatidylserine (DPPS),

20   dimyristoylphosphatidylserine (DMPS), distearoylphosphatidylserine (DSPS), dipalmitoylphosphatidylethanolamine (DPPE), dimyristoylphosphatidylethanolamine (DMPE), and distearoylphosphatidylethanolamine (DSPE). The preferred sterol is cholesterol. Further suitable ingredients of the lipid component are oils, saturated and unsaturated lipid mixtures, glycolipids, and other lipid adjuvants, such as monophosphoryl

25   lipid A (MPLA) or Lipid A. Preferred alcohols include lower alkanols such as ethanols, methanols, propanols, and butanols (i.e., iso-butanol, sec-butanol, and tert-butanol).

To prepare the first solution, a powdered lipid mixture can be dissolved in a minimal amount of alcohol (typically ethanol) with or without water. The water content preferably is

30   less than about 10% of the total volume. The lipid concentration usually is at least about 30 mg/mL, but may be between 5-80 mg/mL. Cholesterol percentage, preferably 0-50 Mol% cholesterol, can be adjusted for optimal entrapment, control of liposome size, and

pharmaceutical activity, as determined empirically by the following examples. Optionally, one or more pharmaceutical substances may be added at this point to the lipid/alcohol mixture. Suitable pharmaceutical substances used in the invention may include proteins, peptides, and biologicals.

5

Preferred proteins include an antibody or an antibody fragment. Preferred peptides include lipopeptides, glycopeptides, and glycolipopeptides. A biological may also refer to DNAs or RNAs, such as intact DNA or mRNA molecules, as well as nucleosides, nucleotides, and oligo and polynucleotides. In addition, biologicals may refer to synthetic or
10   natural organic molecules, vitamins, steroids, immunomodulators, drugs such as antibiotics, anti-tumor agents, anti-inflammatory agents, anti-angiogenesis agents, chemokines, drugs acting on the central nervous system, adjuvants, and infectious disease agent antigens, such as bacteria, viruses, and parasites.

15   A brief sonication bath above the main phase transition temperature (T_m) of the lipid can be used to accelerate dissolution. The main phase transition temperature in the present invention refers to the temperature wherein a typical hydrated lipid transitions from a gel phase with highly ordered hydrocarbon chains to a liquid crystalline phase with disordered hydrocarbon chains. The resultant clear alcohol solution may be sterilized, e.g., by filtration
20   through a 0.22 micron filter.

Second, a separate solution of water-soluble polymer is provided, for example, by mixing the polymer with water under constant stirring. Suitable water-soluble polymers are made from monomers bearing polar groups selected from the groups, e.g., hydroxyl, ether,
25   and amine groups. They include, but are not limited to, polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), and dextran. A preferred water soluble polymer is polyethylene glycol (PEG), typically, PEG 1,000 (MW 1,000), PEG 6,000 (M.W. 6,000), or PEG 20,000 (M.W. 20,000). Polymer concentrations ranging from 10% to about 50% (w/w) are employed. PEGs of high polymerization degree, e.g., PEG 6,000, are employed
30   due to lack of toxicity. Salt content of the PEG solution can be adjusted from 0 to about 10% (w/v), e.g., by adding sodium chloride (NaCl) (preferably between 50 and 154 mM NaCl). This range will be based upon optimal entrapment, resultant liposome size, and

pharmaceutical activity. The polymer solution can be sterilized, for example, by filtration (0.22 micron filter) or by autoclaving. A water–soluble pharmaceutical can be optionally added to the polymer solution.

5   Third, the sterile lipid/pharmaceutical(s) alcohol solution is directly infused into the polymer/pharmaceutical(s) aqueous solution or mixed by directing opposing streams of the alcohol solution to the PEG solution at predetermined volume ratios (*e.g.*, 4:1 to 1:4 Lipid/ alcohol:PEG (v/v)). Basically, the ratio of the lipid to polymer, average M.W. of polymer, polymer concentration, salt content (ionic strength), and temperatures of infusion and

10   incubation are variables in controlling the liposome size, as well as entrapment of a pharmaceutical, as shown in more detail in the Examples below.

        Fourth, the resultant mixture is incubated for a time sufficient to obtain a uniform suspension. Typical incubation times run from at least about 15 minutes to about 24 hours at

15   a temperature ranging from about 4 °C to about 55 °C with intermittent mixing. The mixing and incubation temperature are preferably above the T_m of the lipid mixture to insure good mixing of the components. To ensure the stability of the entrapped pharmaceutical(s), extended exposure to high temperature should be avoided.

20   Fifth, polymer and alcohol typically are removed by washing the liposomes. One method of washing is by addition of sterile water and centrifugation of the mixture, followed by resuspension of the liposomal pellet with sterile water or a physiologically acceptable solution, like normal saline (0.9% (w/v) NaCl). The centrifugation and resuspension are repeated until satisfactory removal of the polymer and alcohol are achieved. The final pellet

25   is resuspended in a physiologically acceptable solution to reach the desired final lipid concentration. This concentration is dependent on the formulation, but is preferably between about 15 to 50 mg/mL bulk lipid (w/v). Washing can also be achieved by standard diafiltration methods under aseptic conditions. The final liposomes are multilamellar vesicles (MLVs) with pharmaceuticals incorporated. The typical average size of the MLVs ranges

30   from one to eight microns depending on the parameters employed.

The instant invention of making larger multilamellar liposomes is simpler, safer, and different from the prior art that is based on the infusion of lipids dissolved in ethanol (Batzri and Korn, 1973; and Kremer et al., 1977) or a water-immiscible solvent (Coe et al., 1996) into water or aqueous buffer. The latter procedures yield predominantly small unilamellar

5   vesicles (SUVs) of mean diameter about 25 nm to 200 nm. In addition, the multilamellar vesicles (MLVs) prepared by the instant invention typically have a mean diameter that ranges from one to eight microns, which is larger than those obtained by Coe et al. (1996) or Leigh (1991).

10   Not wishing to be bound to a particular theory, it is believed that the presence of the polymer causes lipid molecules to coalesce into large multilamellar vesicles. The replacement of alcohol, with the excess bulk water and bound water in the polymer solution, causes the lipid molecules to become immediately hydrated and to spontaneously form a large bilayer structure. The small volume of "free" water within the MLVs forces a high

15   entrapment of the pharmaceutical in either the bilayer hydrophobic core or the aqueous space between the lamellae. The size of the resultant MLVs and their high entrapment of pharmaceuticals make them good candidates for delivery of therapeutic agents.

Liposomes prepared according to the above invention can be used in a wide variety of

20   applications, particularly in inducing potent *in vivo* immunity to incorporated antigen. Liposomes are now employed in numerous immunization procedures. The present liposomes may be substituted into those procedures. Specifically, this method has also been applied to the manufacture of vaccines.

25   Nearly any type of antigen may be incorporated in any of the present liposomes, *e.g.*, for the manufacture of vaccines, including tumor antigens. DNA and RNA encoding antigens may also be used. Tumor antigens may be derived from lung cancers, colon cancers, melanomas, neuroblastomas, breast cancers, ovarian cancers, and the like. A preferred tumor antigen is MUC-1 and its related antigenic peptides and their derivatives. MUC-1 mucin is a

30   high molecular weight glycoprotein with a protein core consisting of tandem repeats of a 20-amino acid sequence and highly-branched carbohydrate side chains. Underglycosylated MUC-1 protein is secreted and abundantly over-expressed in many human adenocarcinomas,

such as breast, colon, lungs, ovarian, and pancreatic cancers. An enhanced level of MUC-1 expression is associated with high metastatic potential and poor prognosis. Thus, MUC-1 is a clinically significant marker for these cancers. Particularly useful antigenic MUC-1 peptide derivatives are based on the 20-amino acid repeat sequence, which may be lipid-modified

5   and/or carbohydrate-modified according to standard methods.

A preferred MUC-1 peptide is BP1-148, a 25-mer antigenic MUC-1-derived lipopeptide, as given by the following sequence:

NH$_2$-[STAPPAHGVTSAPDTRPAPGSTAPP(K-palmitoyl)G]-COOH

10 Two non-MUC-1 amino acids are added as a scaffold for attaching the lipid tail, making a total of 27 amino acids. BP1-148 has a palmitoyl group at the epsilon amino group of the C-terminal lysine. This lipopeptide is the immunogen in a liposomal vaccine (BLP-25) currently in clinical trials for several different types of cancer. Forms of BP1-148, both with and without a lipid tail, are described in Agrawal, B. *et al.*, U.S. Application No. 09/074,410,

15 filed on May 8, 1998, and Boni, L. *et al.*, U.S. Application No. 09/231,641, filed on January 15, 1999, respectively, which are hereby incorporated by reference.

A liposomal vaccine formulation according to the invention may also comprise one or more immunomodulators. An immunomodulator is any substance that alters an immune response, and preferably, stimulates a response to antigen. Typical immunomodulators

20 include but are not limited to adjuvants such as monophosphoryl Lipid A (MPLA), Lipid A, synthetic Lipid A and variants, muramyl dipeptides, CpG oligo's, and lipoteichoic acid. MPLA has been shown to serve as an effective adjuvant to cause increase presentation of lysosomal antigen by the antigen presenting cells to specific T lymphocytes (Alving, C.R.

25 1993). Other immunomodulators include cytokines and lymphokines, especially interleukin-2 (IL-2), chemokines, and costimulatory molecules, especially CD28, CD40, CD40 Ligand, CD80, and CD86.

The following are examples of the present process, using infusion into PEG. They are

30 given to indicate the many parameters that can be varied to help manufacture an optimal liposomal pharmaceutical. These examples are provided to illustrate the present invention and should not be construed to limit the scope of the present invention.

EXAMPLES

5   **Example 1**   Effects of water and PEG infusion methods on liposome size.

This example demonstrates that a liposome of a larger mean liposome size can be obtained by direct infusion of the lipid mixtures into an aqueous PEG solution. A smaller size liposome was obtained with water as the infusion media.

10

A lipid formulation (17.44 mg of DPPC, 2.18 mg of DMPG, and 10.4 mg of cholesterol (50 Mol%)) was dissolved in 0.78 mL of ethanol and 0.04 mL of deionized water. Sonication was performed briefly at 55 $^{\circ}$C in a bath sonicator. The clear solution was then infused into 3.18 mL of either deionized water or a 50% (w/w) PEG 6,000 in deionized water.

15   The mixtures were held at 25 $^{\circ}$C for 1 hour, then diluted to 50 mL with normal saline (0.9% NaCl (w/v)) and subsequently pelleted by centrifugation for 30 min at 18,000 rpm and 4 °C (Sorvall SS-34 rotor). The pellets were washed three times by resuspension into 50 mL normal saline and centrifuged as described above. The final pellets were resuspended in 1.5 mL normal saline.

20

The mean liposome size was 0.4 μm for the sample infused in water, whereas the same lipid mixture infused into 50% PEG in water resulted in a mean liposome size of 2.12 μm (Table 1). This shows a marked increase in the mean diameter of liposomes formed as a result of infusion into a PEG solution over those infused in water, keeping other parameters

25   such as lipid composition, hydration solvent, and infusion volume constant.

Table 1 Sizes of Cholesterol-Containing Liposomes Prepared by Infusion Methods

Example	Sample Composition (Molar)	Infusion Media	Liposome Size (μm)
1	DPPC:CHOL:DMPG 44.1:50.0:5.9	H₂O	0.4
1	DPPC:CHOL:DMPG 44.1:50.0:5.9	50% PEG 6,000	2.1

2	DPPC:CHOL 50.0:50.0	50% PEG 6,000	1.4

Example 2   Effect of lipid composition on liposome size.

5   This example shows that the lipid composition can affect the mean liposome size. The lipid formulation in Example 1 was modified to 19.74 mg of DPPC and 10.4 mg of cholesterol (*i.e.*, DMPG was excluded). Liposomes were prepared in the same way as in Example 1, by infusion into 50% PEG 6,000 in water. The resulting mean liposome size was 1.40 μm as compared to 2.12 μm for the PEG infusion formulated in Example 1 (Table 1).

10   This demonstrates the strong dependence of liposome size on the lipid composition.

         Cholesterol possesses a planar steroid ring which is conformationally inflexible and water insoluble. Hence, the addition of cholesterol orders the lipid bilayer by restricting the conformational flexibility of the lipid hydrocarbon chain and favors a more planar (less

15   curvature) bilayer. The result is a decrease in bilayer packing defects which decreases passive permeability (*i.e.*, reduces solute leakage) and the formation of larger liposomes (planar).

Example 3   Requirements in the generation of large liposomes.

20

         This example demonstrates that generation of large liposomes is not solely dependent on the different lipid mixtures being added but it is also dependent on the varying PEG concentrations being used.

25   Figure 1 compares mean liposomal sizes obtained when formulations from Examples 1 and 2 were infused into 0, 10%, 25%, and 50% (w/w) PEG solutions at 25 °C. As can be seen, different lipid mixtures have different PEG concentration requirements for generating large liposomes.

30   **Example 4**   Effect of infusion temperature on mean liposome size using 50% PEG 6,000.

-11-

This example shows that at a higher temperature, a larger liposome can be obtained with 50% PEG 6,000.

5   The same preparation was made as in Example 2. The PEG infusion and incubation temperature was 55 $^{\circ}$C instead of 25 $^{\circ}$C. The mean liposome size obtained was 1.94 μm at 55 $^{\circ}$C compared with 1.52 μm at 25 $^{\circ}$C (Figure 2). Thus, temperature can be raised to obtain a larger liposome. It can also be lowered to obtain a liposome of a smaller size.

10   **Example 5**   Effect of PEG polymerization on mean liposome size at 25 °C and 55 °C.

This example demonstrates that liposome size is directly dependent on the molecular weight of PEG.

15   The same preparation was made as in Example 2 with 50% PEG 20,000 instead of PEG 6,000. The mean liposome size was 3.00 μm at 25 $^{\circ}$C and 3.97 μm at 55 $^{\circ}$C. Comparing these results to the liposome sizes obtained with PEG 6,000 (Figure 3), it appears that infusion into the higher MW PEG yields a larger liposome.

20

Example 6   Effect of salt concentration on the final liposome size.

This example illustrates the influence of ionic strength of the PEG solution on the final liposome size and its dependence on lipid composition.

25

The same preparations were made as in Examples 1 and 2, but infused into 50% PEG 6,000 in 10% NaCl. As shown in Figure 4, a 25 °C infusion into PEG 6,000 in high ionic strength media resulted in mean liposome sizes of 2.48 μm (w/DMPG) and 1.68 μm (w/o DMPG). In contrast, similar preparations infused into PEG 6,000 in low ionic strength

30   solvent gave smaller liposome sizes of 2.12 μm and 1.4 μm, respectively (Table 1). At 55 $^{\circ}$C, consistent with the temperature effect noted in Example 4, infusion into PEG 6,000 in high ionic strength media further increased the mean liposome sizes to 3.69 μm (w/DMPG) and

4.12 µm (w/o DMPG). Thus, the ionic strength of the PEG solution can affect the final liposome size.

Example 7   Effect of infusion into increased volumes of PEG 6,000 in 10% (w/v) NaCl
5   solution on liposome size..

This example shows a decrease in mean liposomal size with infusion into higher volumes of PEG 6,000 in 10% (w/v) NaCl.

10   In the manner of Examples 1 and 2, formulations were prepared with and without DMPG in 0.78 mL of ethanol and 0.04 mL of deionized water. These formulations were infused into 3.2 mL, 1.6 mL, 0.8 mL, and 0.4 mL of a 50% PEG 6,000 solution containing 10% NaCl at either 25 °C or 50 °C. Figure 5 exhibits a decrease in mean liposomal size as the ratio of PEG solution:Infusate (v/v) increases. This effect is observed at 25 °C and 50°C
15   and appears independent of DMPG inclusion.

Example 8   Influence of varying cholesterol content on the effects of PEG and saline infusions on liposome size.
20

A lipid formulation of 19.75 mg of DPPC with cholesterol varying from 0 mg to 10.4 mg (0 to 50 Mol%) was dissolved in 0.78 mL ethanol and 0.04 mL water. Large liposomes were formed following both PEG (in 10% NaCl) and saline (w/o PEG) infusion. Figure 6 shows that, with increased cholesterol content, saline infusion exhibited a decrease in vesicle
25   size. In contrast, PEG infusion demonstrated an increase in vesicle size with increasing cholesterol content. This indicates that PEG infusion is more appropriate for making larger vesicles at high cholesterol content.

Example 9   Induction of peptide-specific cellular responses by liposomal vaccines
30   prepared from direct PEG infusion.

The objective of this Example is to illustrate that liposomal vaccines prepared from direct infusion of PEG are capable of stimulating antigenic responses.

5   Sample FW91699-1 was prepared by infusion of an ethanol solution containing DPPC, cholesterol, Lipid A, and BP1-148 into 50% (w/w) PEG 6,000 in 10% (w/v) NaCl . . Sample FW91699-2 was prepared in a similar manner but with infusion into PEG 20,000 in water. The PEG was removed by washing with normal saline followed by centrifugation to obtain the pelleted MLVs. These were compared to a control BLP25 vaccine formulation, BUSA 27, currently in clinical trials. BUSA27 was prepared by first forming LUVs by
10   ethanol infusion, followed by lyophilization and reconstitution in sterile normal saline solution to make MLVs. BUSA 27 also contains DPPC, cholesterol, Lipid A, and BP1-148, but in addition has 5.9 Mol% DMPG.

Each formulation was injected subcutaneously into the inguinal region of C57BL/6
15   mice. Nine days after injection, spleens and lymph nodes were removed and dissociated into cells. Cells were cultured for 72 hours with peptide BP1-151, a nonpalmitated version of BP1-148, with the negative control peptide BP-072 or with medium. Proliferation of lymph node cells was determined by Alamar Blue assay and release of IFN-γ was determined by a capture Ab assay.
20

Table 2 shows that the liquid formulations, FW91699-1 and FW91699-2, induced peptide-specific (BP1-151) immune responses in mice.

Table 2 Proliferation and IFN-γ Results

25   Proliferation Assay

Treatment	Optical Density 570/610 nm			
	Medium	BP-072	BP1-151	BP1-151 Peptide Specific Proliferation
Saline	0	0.007	0	0
BUSA 27	0.176	0.079	0.543	0.367
FW91699-1	0.02	0	0.410	0.390
FW91699-2	0.08	0	0.405	0.325

IFN-γ Assay   Lymph Nodes

Treatment	Interferon gamma (ng / mL)			
	Medium	BP-072	BP1-151	BP1-151 Peptide Specific IFN-γ Release
Saline	0.7	0.7	0.7	0
BUSA 27	3*	3*	59*	56
FW91699-1	3*	3*	49*	46
FW91699-2	3*	3*	51*	48

*1:5 dilution all the remaining supernatants were tested at a 1:2 dilution.

<u>IFN-γ Assay Spleen Cells</u>

Treatment	Interferon gamma (ng / mL)			
	Medium	BP-072	BP1-151	BP1-151 Peptide Specific IFN-γ Release
Saline	0.7	0.7	0.7	0
BUSA 27	3*	3*	114*	111*
FW91699-1	3*	3*	90*	87*
FW91699-2	3*	3*	85*	82*

*1:5 dilution all the remaining supernatants were tested at a 1:2 dilution.

Example 10   Proliferation of T Cells and γ-interferon production by different BLP-25-like

5   liposomal vaccine formulations.

This example provides further evidence that BLP-25-like liposomal vaccines
formulated from direct PEG infusion can stimulate cellular immune responses by inducing T
cell proliferation and IFN-γ production.

10

Four liquid formulations of BLP-25 vaccine prepared by the PEG-infusion method
were manufactured by controlling temperature, infusion rate, and mixing speeds. These were
compared to standard preparations denoted BUSA 014 and BUSA 26, two additional lots
prepared like BUSA 27 (see Example 9). Both standards are manufactured following the
15   procedure for BUSA 27 and contain DPPC, cholesterol, Lipid A, BLP-148, and 5.9 Mol%
DMPG. Infusion was into saline alone or 50% PEG 6,000 in 10% NaCl. The components of
the formulations were DPPC (11.6 mg/mL), cholesterol (6.9 mg/mL), BP-148 (400 μg/mL),
and Lipid A (200 μg/mL), with and without DMPG as noted in Table 3. These formulations
were washed by diafiltration at a constant inlet pressure of 15-20 psi against sterile-filtered
20   phosphate buffered saline (10 mM sodium phosphate, 0.9% (w/v) NaCl, pH 6.6). Infusion
into PEG and inclusion of DMPG both contributed to a higher recovery of the BP1-148
peptide (Table 3).

Table 3   Infusion Into Different Media

Liquid Formulations	Mixing Medium	DMPG (1.5 mg/mL)	Recovery of BP1-148 (µg/mL)
BLP-25-1	normal saline (0.9% NaCl)	+	204
BLP-25-2	50% PEG in 10% NaCl	+	318
BLP-25-3	normal saline (0.9% NaCl)	–	123
BLP-25-4	50% PEG in 10% NaCl	–	243

These formulations were injected into C57BL/6 mice (subcutaneous, inguinal region) to determine their potency for induction of peptide-specific immune responses (proliferation of T cells and IFN-γ production). Figure 7A demonstrates that all four formulations induced a BP1-148 specific proliferative response in T cells from the draining lymph nodes which was comparable to that induced by standard preparations BUSA 26 and BUSA 014. Figure 7B shows that all four liquid formulations and the standard preparations, BUSA 26 and BUSA 014, also primed T cells to produce significant amounts of IFN-γ in response to restimulation in vitro with BP1-148. The amount of IFN-γ released by the cells roughly correlated with the recovery of the BP1-148 peptide in the individual formulations (Table 3).

REFERENCES

Abra, R.M., Hunt, C.A. and Lau, D.T. (1984) Liposome disposition in vivo VI: delivery to the lung. J. Pharm. Sci. 73:203-206.

5

Agrawal, B. *et al.,* (1988) U.S. Application No. 09/074,410.

Allen, T.M., and Everest, J.M. (1983) Effect of liposome size and drug release properties on pharmacokinetics of encapsulated drug in rats. J. Pharm. Exp. Therapeutics 226:539-544.

10

Alving, C.R. (1993) Lipopolysaccharide, lipid A, and liposomes containing lipid A as immunologic adjuvants. Immunobiology 187:430-446.

Batzri, S., and Korn, E.D. (1973) Single bilayer liposomes prepared without sonication.

15 Biochim. Biophys. Acta. 298:1015-1019.

Boni, L.T., Stewart, T.P., Alderfer, J.L., and Hui, S.W. (1981) Lipid-polyethylene glycol interactions: I. Induction of fusion between liposomes. J. Membrane Biol. 62:65-70.

20 Boni, L.T., Hah, J.S., Hui, S.W., Mukherjee, P., Ho, J.T., and Jung, C.Y. (1984) Aggregation and fusion of unilamellar vesicles by poly(ethylene glycol). Biochim. Biophys. Acta 775:409-418.

Boni, L. *et al.,* (1999) U.S. Application No. 09/231,641.

25

Coe, R.M., Edgerly-Pflug, L., Boni, L., Portnoff, J., and Sharma, M.L. (1996) Unites States Patent 5,540,936.

Fidler, I.J., Ras, A., Fogler, W.E., Kirsh, R., Bugelski, P., and Poste, G. (1980) Design of

30 liposomes to improve delivery of macrophage-augmenting agents to alveolar macrophages. Cancer Res. 40:4460-4466.

Gregoriadis, G., Neerunjun, D.E., and Hunt, R. (1977) Life Sci. 21:357.

Harashima, H., Ohnishi, Y., and Kiwada, H. (1992) In vivo evaluation of the effect of the size and opsonization of the hepatic extraction of liposomes in rats: an application of the Oldendorf method. Biopharm. Drug Disp. 13:549-553.

Harris, J.M. (1992) Introduction to the biotechnical and biomedical applications of poly(ethylene glycol). In: Poly(ethylene glycol) chemistry, biotechnical and biomedical applications, ed. Harris, J.M., pp. 1-14, Plenum Press, New York.

Heath, T.D., Lopez, N.G., and Papahadjopoulos, D. (1985) The effects of liposome size and surface charge on liposome-mediated delivery of methotrexate-aspartate to cells in vitro. Biochim. Biophys. Acta, 820:74-84.

Hui, S.W., Stewart, T.P., Boni, L.T., and Yeagle, P.L. (1981) Membrane fusion through point defects in bilayers. Science 212:921-923.

Hunt, C.A., Rustum, Y.M., Mayhew, E., and Papahadjopoulos, D. (1979) Retention of cytosine arabinoside in mouse lung following intravenous administration in liposomes of different size. Drug Metab. Dispos. 7:124-128.

Jackson, A.J. (1981) Intramuscular absorption and regional lymphatic uptake of liposome-entrapped inulin. Drug Metab. Dispos. 9:535-540.

Jackson, A.J. (1980) The effect of route of administration of the disposition of inulin Encapsulated in multilamellar vesicles of defined particle size. Res. Comm. Chem. Path. Pharm. 27:293-304.

Kremer, J.M., Esker, M.W., Pathmamanoharan, C., Wiersema, P.H. (1977) Vesicles of variable diameter prepared by a modified injection method. : Biochemistry 17:3932-5.

Leigh, S. (1991) United States Patent 5,053,217.

Loughrey, H.C., Wong, K.F., Choi, L.S., Cullis, P.R. and Bally, M.B. (1990) Protein liposome conjugates with defined size distributions. Biochem. Biophys. Acta 1028:73-81.

5   Moghimi, S.M., Porter, C.J., Muir, I.S., Illum, L., and Davis, S.S. (1991) Non-phagocytic uptake of intravenously injected microspheres in rat spleen: influence of particle size and hydrophilic coating. Biochem. Biophys. Res. Commun. 177:861-866.

Poste, G., Kirsh, R., and Koestler, T. (1984) The challenge of liposome targeting in vivo.
10   In: Liposome Technology, ed., G. Gregoriades, Volume III, pp. 1-28, CR. Press.

Proffitt, R.T., Williams, L.E., Presant, C.A., Tin, G.W., Uliana, J.A., Gambe, R.C., and Baldeschwieler, J.D. (1983) Liposomal blockade of the reticuloendothelial system: improved tumor imaging with small unilamellar vesicles. Science 220:502-505.

15

Rahman, A., Fumagalli, A., Barbieri, B., Schein, P.S., and Casazza, A.M. (1986) Antitumor and toxicity evaluation of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes. Cancer Chem. Pharm. 16:22-27.

20   Rahman, Y.E., Cerny, E.A., Patel, K.R., Lau, E.H., and Wright, B.J. (1982) Differential uptake of liposomes varying in size and lipid composition by parenchymal and Kupffer cells of mouse liver. Life Sci. 31:2061-2071.

Suzuki, S., Ohta, S., Takashio, K., Nitanai, H., and Hashimoto, Y. (1990) Int. J. Cancer
25   46:1095-1100.

Working, P.K., Newman, M.S., Johnson, J., and Cornacoff, J.B. (1077) Safety of poly(ethylene glycol) and poly(ethylene glycol) derivatives. In Poly(ethylene glycol) chemistry and biological applications, eds., Harris, J.M. and Zalipsky, S., pp. 45-57.
30   American Chemical Society, Washington, D.C.

CLAIMS

1. A method of preparing large liposomes comprising:

   (a) providing a first solution comprising a lipid component in a water-miscible
   alcohol or an alcohol/water mixture,

   (b) providing a second solution of water-soluble polymer,

   (c) infusing the first solution directly into the second solution thereby forming a
   liposome suspension, and

   (d) removing the alcohol and polymer from the suspension to produce the desired
   large liposomes.

2. The method of Claim 1, wherein said alcohol and polymer are removed by washing
the mixture through centrifugation or diafiltration.

3. The method of Claim 1, wherein said lipid component is selected from the group
consisting of a phospholipid, an oil, and a mixture of saturated and unsaturated lipids.

4. The method of Claim 1, wherein said lipid component comprises at least one lipid and
a sterol.

5. The method of Claim 1, wherein the lipid/alcohol solution is an emulsion.

6. The method of Claim 3, wherein the phospholipid is selected from the group
consisting of phosphatidylcholine, phosphatidylglycerol, phosphatidic acid,
phosphatidylserine, and phosphatidylethanolamine, dipalmitoylphosphatidylcholine,
distearoylphosphatidylcholine, dimyristoylphosphatidylcholine,
dipalmitoylphosphatidylglycerol, distearoylphosphatidylglycerol,
dimyristoylphosphatidylglycerol, dipalmitoylphosphatidic acid; dimyristoylphosphatidic acid,
distearoylphosphatidic acid, dipalmitoylphosphatidylserine, dimyristoylphosphatidylserine,
distearoylphosphatidylserine, dipalmitoylphosphatidylethanolamine,
dimyristoylphosphatidylethanolamine, and distearoylphosphatidylethanolamine.

7.   The method of Claim 4, wherein the sterol is a cholesterol.

8.   The method of Claim 1, wherein the molecular weight of the polymer is from about 6,000 Dalton to about 20,000 Dalton.

5

9.   The method of Claim 1, wherein said polymer is made from monomers bearing polar groups selected from the group consisting of hydroxyl, ether, and amine groups.

10.   The method of Claim 1, wherein the polymer is selected from the group consisting of
10   polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), and dextran.

11.   The method of Claim 1, wherein the water content of the first solution is about 0 to about 10% of the total volume.

15

12.   The method of Claim 1, wherein the lipid concentration of the first solution is at least about 10 mg/mL.

13.   The method of Claim 1, wherein the polymer concentration is at least about 25%
20   (w/w).

14.   The method of Claim 1, wherein the average size of the liposome ranges from about one micron to about 8 microns.

25   15.   The method of Claim 1, further comprising adding at least one pharmaceutical substance to either the first or the second solution, or to both.

16.   The method of claim 15, wherein the at least one pharmaceutical substance is selected from the group consisting of a protein, a peptide, an antibody, an antibody fragment, a
30   biological, a tumor antigen, and a mixture thereof.

17.   The method of Claim 16, wherein said peptide is a lipopeptide, a glycopeptide, or a glycolipopeptide.

18.   The method of Claim 16, wherein said biological is selected from the group consisting of DNAs, RNAs, nucleosides, nucleotides, oligonucleotides, polynucleotides, synthetic organic molecules, natural organic molecules, vitamins, steroids, immunomodulators, cytokines, lymphokines, costimulatory molecules, antibiotics, antitumor agents, anti-inflammatory agents, anti-angiogenesis agents, chemokines, adjuvants, drugs acting on the central nervous system, an infectious disease agent antigen, and a mixture thereof.

19.   The method of Claim 16, wherein the biological is interleukin-2.

20.   The method of Claim 16, wherein the biological is an infectious disease agent antigen selected from the group consisting of a bacteria, a virus, a parasite, and a mixture thereof.

21.   The method of Claim 16, wherein the tumor antigen is a peptide.

22.   The method of Claim 21, wherein the peptide is a MUC-1 peptide or a derivative thereof.

23.   The method of Claim 22, wherein the MUC-1 peptide is glycosylated.

24.   The method of claim 22, wherein the MUC-1 peptide is BP1-148, having the following sequence:

NH_2-[STAPPAHGVTSAPDTRPAPGSTAPP(K-palmitoyl)G]-COOH

25.   Liposomes prepared according to the method of claim 1.

26.   A cancer vaccine, comprising liposomes prepared by the method of Claim 1 and a tumor antigen.

27.   A cancer vaccine comprising at least one phospholipid, cholesterol, lipid A (natural or synthetic), and a tumor antigen.

28.   An infectious disease agent vaccine comprising liposomes prepared by the method of Claim 1 and an infectious disease agent antigen.

29.   The vaccine of Claim 28, wherein the infectious disease agent antigen is selected from the group consisting of a bacteria, a virus, a parasite, and a mixture thereof.

Figure 1[*]

Liposome Size vs. PEG Concentration
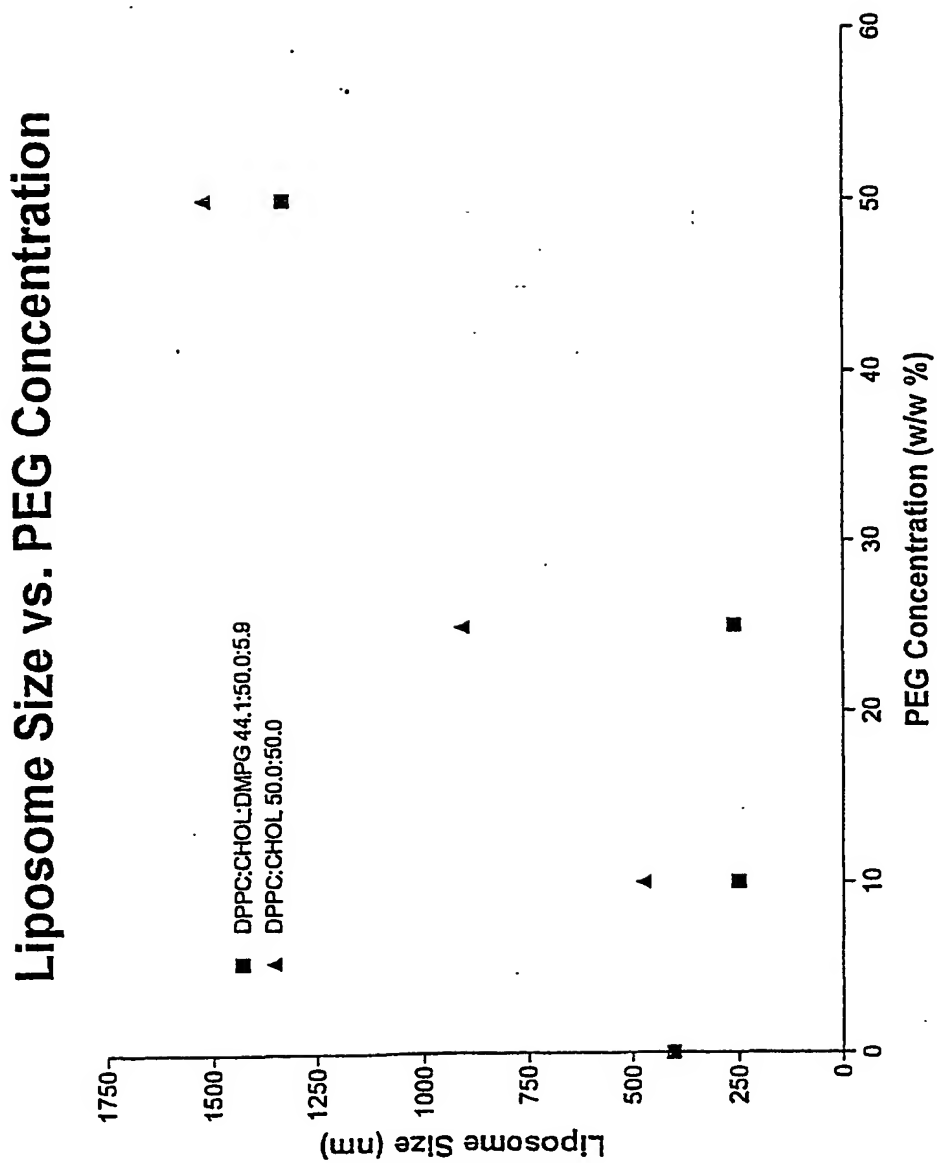
* See Example 3.

*Figure 2**

Liposome Size Affected by Infusion Temperature

DPPC:CHOL 50.0:50.0
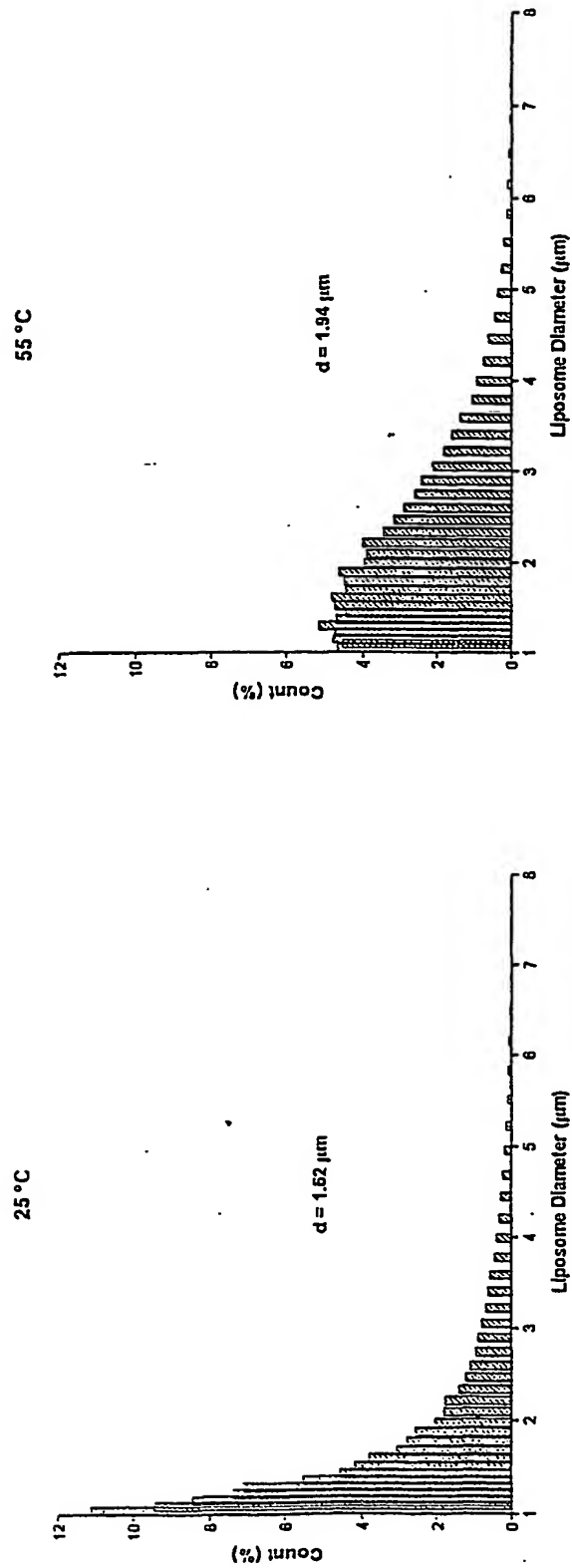
* See Example 4.

Figure 3 *

* See Example 5.

Figure 4[*]

Size of Liposomes by PEG 6000 (10 % NaCl) Infusion

DPPC:CHOL:DMPG 44.1:50.0:5.9
DPPC:CHOL 50.0:50.0
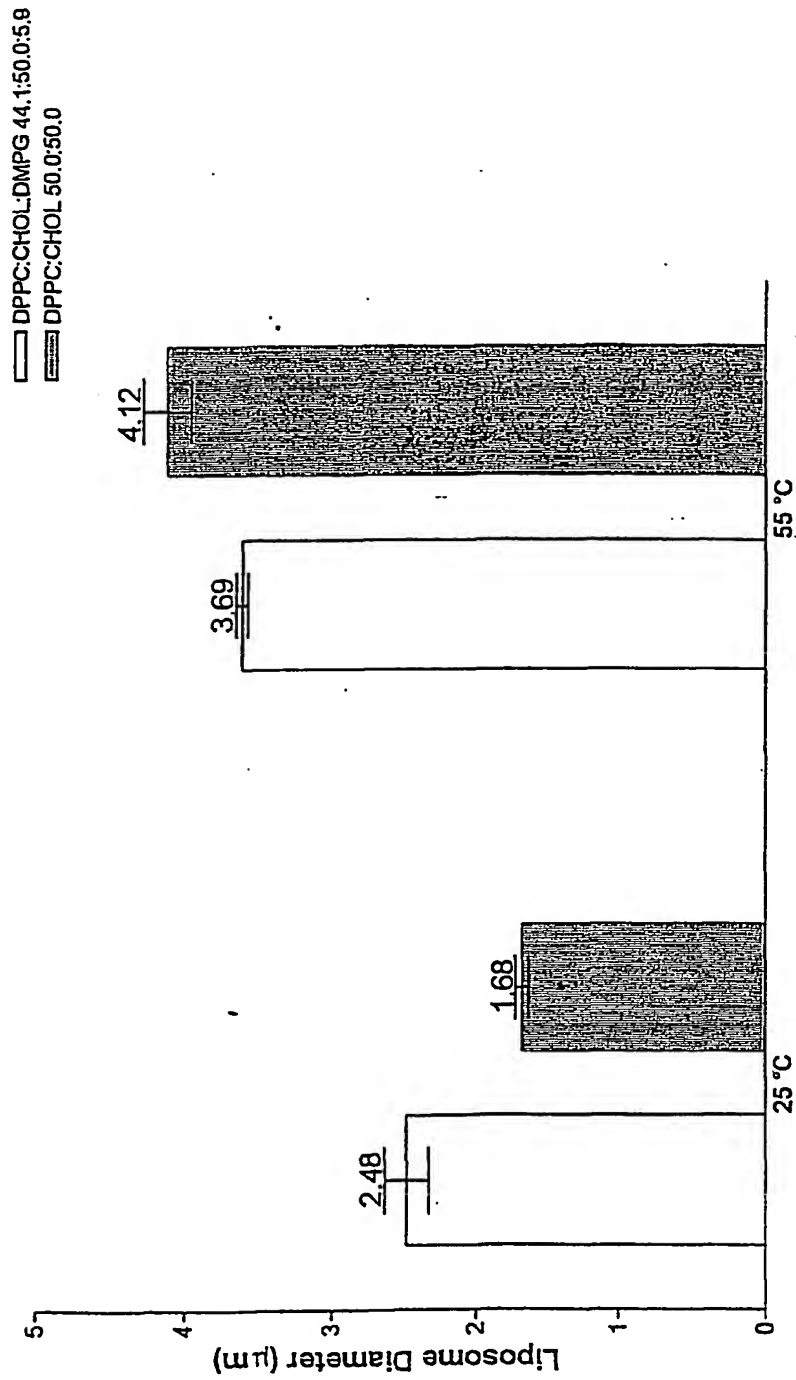
* See Example 6.

Figure 5*

Liposome Size vs. PEG Amount:
PEG6000 + NaCl

Legend:
- DPPC:ChOL:DMPG 44.1:50.0:5.9, 25 °C
- DPPC:CHOL 50.0:50.0, 55°C
- DPPC:CHOL:DMPG 44.1:50.0:5.9, 55 °C
- DPPC:CHOL:DMPG 50.0:50.0, 55 °C

V_{PEG} / V_{EtOH}

Particle Size (μm)
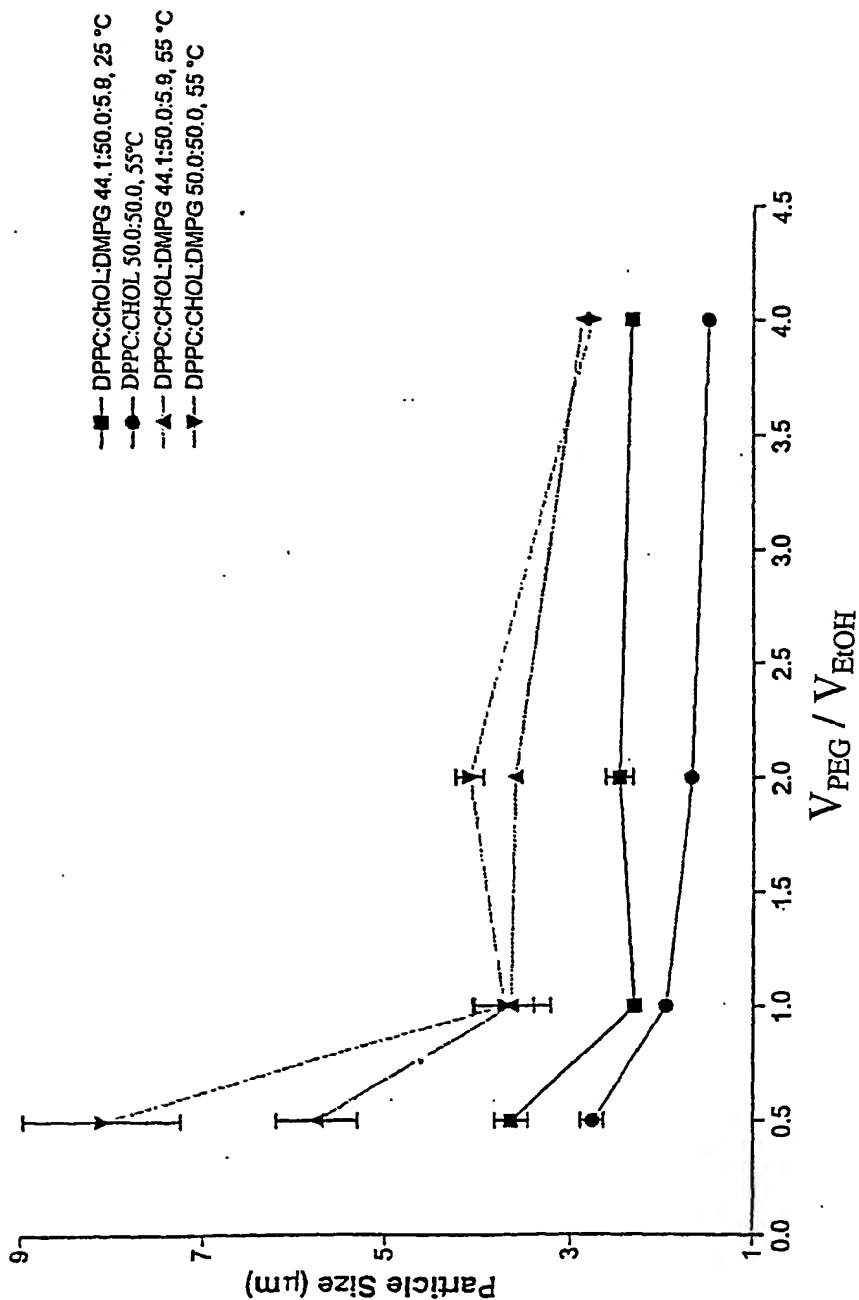
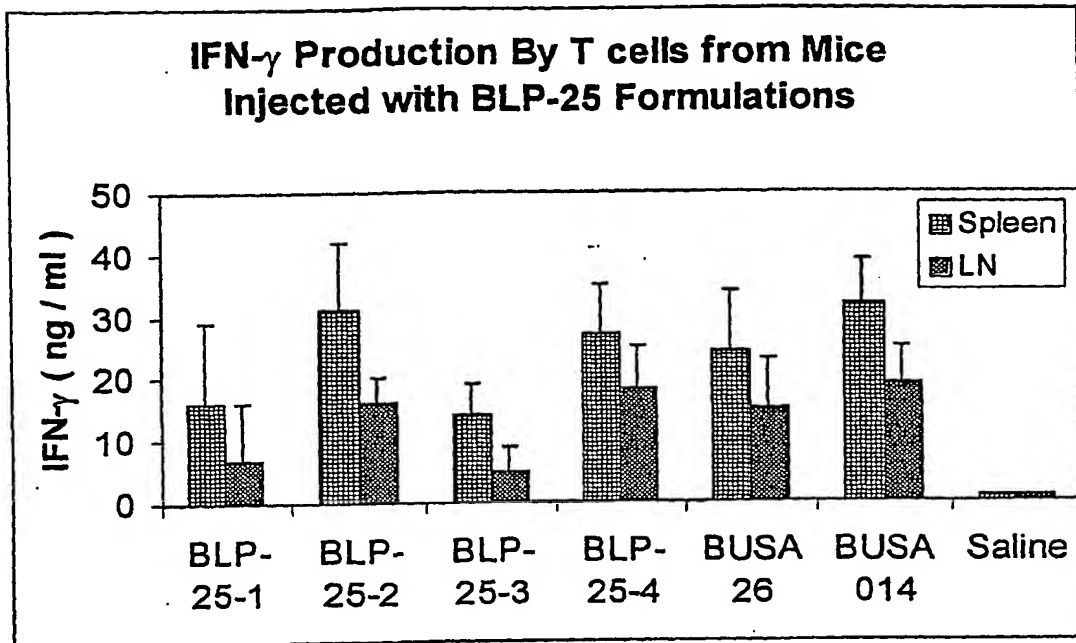* See Example 7.

Figure 6

Cholesterol Titration
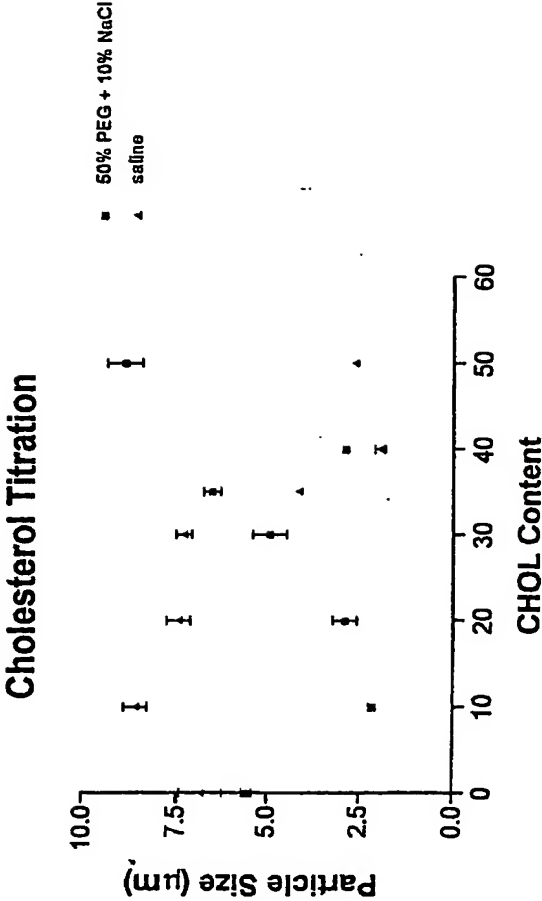
(see example 8)

Figure 7A
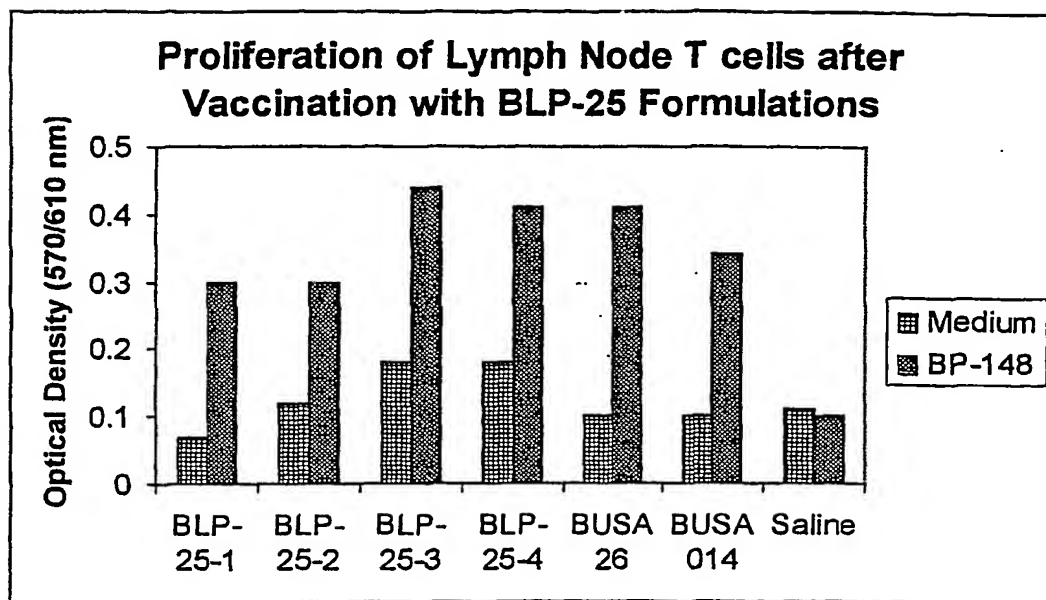
(see example 10)

Figure 7B

(see example 10)